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14. ABSTRACT The *NF1* that is highly expressed in developing neural cells and loss-of-function mutations in *NF1* gene results in deregulation of Ras signaling which contributes to cancer development. We reported that hyperactive Ras could induce apoptosis after inhibiting PKC which was not harmful to normal, cells. To define the role of PKC isoforms, we identified the sequences of shRNAs targeting each PKC isoform. With the aid of PKC inhibitor and shRNAs, we successfully define that the concurrent suppression of PKC alpha/bete lethally interacts with hyperactive Ras in NF1 deficient cells. We also cloned and inserted NF1 effector domain gene into an expression vector tagged with flag and introduced it into NF1 deficient cells that became less sensitive to loss of PKC. Various Ras loop mutant genes were introduced into NF1 deficient cells. Subsequently, the experiments using the mutants and inhibitors demonstrate the involvement of PI3K/Akt and JNK, as Ras downstream effectors, in transmitting the apoptotic signaling. The data that generated through the funding period reached the aims proposed in this grant application and indicate the feasibility of our hypothesis. Now, the completion of the project gives a comprehensive understanding of the synthetic lethal interaction between aberrant Ras in NF1 deficient cells and loss of PKC. However, to elucidate the mechanisms of this synthetic lethality requests further and thorough investigation.

15. SUBJECT TERMS
NF1, Apoptosis, Synthetic lethality, PKC isoforms, Aberrant Ras.

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INTRODUCTION

Children with neurofibromatosis typically develop central nervous system abnormalities, including aberrant proliferation of astrocytes. Among the various manifestations of neurofiromatosis type 1 (NF1), malignant peripheral nerve sheath tumor (MPNST) represents a major challenge in clinical treatment, because the patients response poorly to therapeutic modalities. The *NF1* gene encodes neurofibromin (a Ras-GAP) that is highly expressed in developing neural cells and loss-of-function mutations in *NF1* gene results in deregulation of Ras signaling which contributes to cancer development (1). Whereas loss-of-function of neurofibromin and subsequent upregulation of Ras pathway have received considerable attention from the perspectives of cancer etiology, the ability of Ras to induce apoptosis and it therapeutic perspectiveness in NF1 cells remain relatively unexplored. We previously reported that Ras not only mediates cell proliferation but also apoptosis (2). We showed that various types of human or murine cells expressing an activated *ras*, after endogenous protein kinase C (PKC) activity/expression being suppressed, underwent apoptosis. In contrast, PKC inhibition is not harmful to normal, control cells that arrested in G_1 phase of the cell cycle (3). This led us to test the feasability of induction of Ras-mediated apoptosis in NF1 through inhibition of PKC. We demonstrated that the addition of GO6796 (a PKC inhibitor) caused a high magnitude of apoptosis in NF1-deficient MPNST and Schwann cells. After suppression of Ras, the loss of PKC could no longer sensitize NF1-deficient cells, indicating the dependency of aberrant Ras signaling for the induction of apoptosis. Furthermore, during this apoptotic process, JNK and PI3K/Akt played the crucial roles. All our data generated in the funding period support the hypothesis that *therapeutic intervention into crucial intracellular signaling, such as in the Ras pathway, can induce apoptosis in NF1-deficient cells. Such an apoptotic process is regulated in the context of other regulatory influences, among them, suppression of PKC activity*. Our long-term goal is to further elucidate the mechanisms of the possibility of the induction of apoptosis in NF1 deficient tumors, the result of which will provide a molecular basis to foster novel strategies for the treatment of the disease.

BODY

Task 1. PKC is a family of various structurally related serine/threonine protein kinases, which consists of more than 12 isoforms and our preliminary data showed that the suppression of PKC by GO6976 elicited apoptosis in the NF1-deficient cells via activation of Ras pathway. Therefore, we will determine which PKC isoform(s), once being suppressed, is/are responsible for induction of Ras-mediated apoptosis in NF1-deficient cells.

a. siRNA against each PKC isoform will be constructed in a retroviral vector.

GO6976 has been recommended by the company to be a specific inhibitor for PKC α and β. Because these two isozymes belong the subgroup of phorbol ester-dependent PKCs, our primary focus was to knock down this group of PKc isoforms. Several different sequences of *shRNAs* at various locations of each PKC isoform were selected and inserted into a lentiviral vector (that is recommended recently by many groups to be the most effective vector) (**Table 1**). After infected the selected *shRNA*s into sNF cells, we identified that the sequence 1 for each PKC isoform listed in table 1 is the best *shRNA* for the knocking-down. The Western blot results of the inhibitory effect on sNF cells using the *shRNA*s with the sequence 1 were presented in **Figure. 1**. RT-PCR was also performed to confirm that the knockdown of each PKC isoform is at the gene level (data not shown).

Table I

PKC Isoform	*shRNA* sequence 1	*shRNA* sequence 2	*shRNA* sequence 3
PKC α	5'-*GAACGTGCATGAGGTGAAA*-3'	5'-ATTCATCGCCCGCTTCTTC-3'	5'-GACAGCCTGTCTTAACACC-3'
PKC β	5'-*GAGATTCAGCCACCTTATA*-3'	5'-TCTGCTGCTTTGTTGTACA-3'	5'-GTCATTTGGGATTTCAGAA-3'
PKC θ	5'-*CGAGAAACCATGTTCCATA*-3'	5'-CAGAACATGTTCAGCAACT-3'	5'-CTAGGAGATGCGAAGACAA-3'
PKC ε	5'-*GCAAGGAAGGGATTATGAA*-3'	5'-ATCACCAACAGTGGCCAAA-3'	5'-CGTTTCTTCGAATCGGTTT-3'
PKC γ	5'-*TCAGAGCTGAAGGCTGGG*-3'	5'-CCAGTGCCAAGTTTGCTGTTT-3'	5'-AACAGGGAGATCCAACCACCA-3'
PKC δ	5'-CTGTTTGTGAATTTGCCTT-3'	5'-CAGAGTTCCTGAATGAGAA-3'	5'-GCATCTCCTTCAATTCCTA-3'
PKC η	5'-GTGAACGGACATAAGTTCA-3'	5'-CTCACCGATTCAAGGTTTA-3'	

Various *shRNA* sequences directed to different PKC isoforms were selected and the sequence 1 is the effective one.

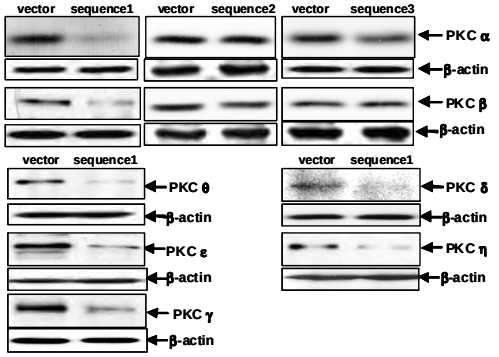

Figure 1. Western blot of various PKC isoform expressions after introduced *shRNA* sequences into sNF cells.

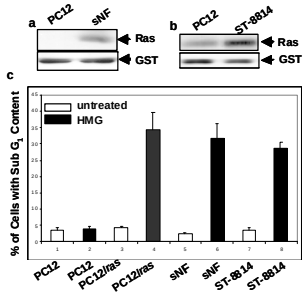

Figure 2. Ras activation and Induction of apoptosis in *Nf1* deficient cells. **a** and **b**. Lysates from sNF and ST-8814 cells were co-immunoprecipitated with a RBD/GST fusion protein and immunoblotting with anti-Ras antibody. Even loading was verified by GST expression. **c**. After treated with HMG (0.5 uM, 48 h), DNA fragmentation in the cells were measured by a flow cytomer. Error bars represent SD over 5 independent experiments.

b. The vector carrying specific siRNA will be infected into NF1-proficient or deficient cells. Subsequently, induction of apoptosis will be determined by DNA fragmentation and ATP assays.

Ras mutations, together with loss of PKC, have been shown to be synthetically lethal in various types of cells (including human lymphoid cells ectopically expressing active

ras or lung cancer cells harboring an oncogenic *ras*). Since Ras signaling pathway in *Nf1* deficient cells is aberrant, it led us to test whether hyperactive Ras signaling could be in synergy with loss of PKC in NF deficient condition. Human sNF (MPNST) and ST-8814 (Schwannoma) cells that are NF1 deficient cells were used. Rat pheochromocytoma PC12 cells were selected as a control, in which Ras signaling is normal. The activity of Ras in these cells was determined by immunoblotting using a RBD/GST fusion protein that contains a Raf interaction domain with Ras and can only associate with the active form of Ras (**Figs. 2a** and **b**). The association of active Ras with the fusion protein was observed in sNF or ST-8814 cells, which confirms that Ras signaling is active in these *Nf1* deficient cells. The susceptibility of these cells to apoptosis in response to HMG (a PKC inhibitor) treatment was tested (**Fig. 2c**). Both *Nf1* deficient cells, as well as PC12 cells ectopically expressing *v-ras*, underwent apoptosis after the suppression of PKC. In contrast, the treatment with HMG was not apoptotic to parental PC12 cells. All the data suggest that aberrant Ras signaling, together with loss of function of PKC, are synthetically lethal in NF1 deficient cells.

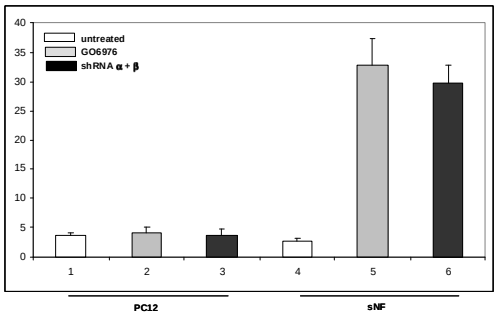

Figure 3. Induction of apoptosis in sNF cells after concurrent suppression of PKC α and β by the *shRNAs* and GO6976 (a PKCα and β inhibitor). DNA fragmentation assay was used and the error bars represent the standard deviation from 5 independent experiments.

Each effective shRNA was also infected in ST cells and the induction of apoptosis was tested. The concurrent knockdown of PKC α and β, but not other PKC isoforms, sensitized sNR, but not PC12 cells, to apoptosis (**Fig. 3**), which indicates the importance of these two PKC isoforms in the support of the survival of NF1 deficient cells.

c. The identified PKC isoform(s) will be overexpressed in the same experimental settings to test if it will desensitize the cells to apoptosis.

After demonstrated the susceptibility of NF1 deficient cells to apoptosis, we then tested whether the cells were less susceptible to Ras-mediated apoptosis when PKC α and β were overexpressed. A few NF1 cells co-overexpressing PKC α and β underwent apoptosis. The results further suggest the role of these two PKC isoforms in the induction of the synthetic lethality.

Some of the results were presented in the manuscripts (entitled, "Synthetic Lethal Interaction between Aberrant Ras and Loss of PKC α/β in *NF1* Deficient Cells") that are being reviewed by Oncogene. Another manuscript to elucidate the mechanisms of this synthetic lethality is being prepared and will be submitted shortly.

Task 2. Ras is a key intracellular signal transducer and regulates multiple downstream effector pathways. Constitutive activation of PI3K/Akt/mTOR pathway has been seen in NF1-deficient cells, especially in MPNST cells. Our preliminary data showed that JNK activity is upregulated during PKC/Ras-mediated apoptosis in the NF-deficient cells. These observations suggest that Ras preferentially uses different downstream effectors under different circumstances (growth proficient conditions vs. loss of PKC) to generate different outcomes. Therefore, we will determine whether

and how JNK functions as Ras downstream effector to transmit the apoptotic signals in NF1-deficient cells.

a. Various ras mutants inserted in a MSCV retroviral vector will be stably or transiently introduced into NF-1 proficeint or deficient Schwann or MPNST cells.

To explore the mechanisms, the cells were infected with *V12ras* effector loop mutant genes. Among these mutants, V12S35Ras is able to bind to Raf; V12C40Ras preferentially activates PI3K; and V12G37Ras interacts with RalGDS only. The expression level of Ras and the loop mutants in the cells with or without treated with GO6976 (a PKC inhibitor) was determined by Western blot. The transfectants expressed high levels of the proteins (**Fig. 3**). The amount of the proteins was not changed by adding GO6976 (data not shown).

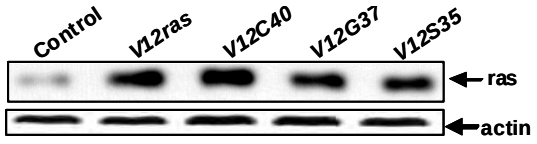

Figure 3.Expression levels of the cells infected with *v-12ras* and different *ras* loop mutant genes by immunoblotting.

b. By suppression or overexpression, how and whether PI3K/Akt/mTOR and JNK cooperate with or antagonize each other in the induction of apoptosis or if other Ras downstream pathways contribute to induction of apoptosis will be determined.

To determine which Ras downstream effectors, in response to PKC inhibition, transmits the apoptotic signaling, DNA fragmentation (**Fig. 4**) and Annexin V assays were conducted (data not shown), respectively. Both assays showed that about 40% of ST cells had fragmented DNA following GO6976 treatment, which was partially blocked by wortmannin (a PI3K inhibitor) and JNK inhibitor. On the contrary, the overexpression of *v12C40* or *JNK* to upregulate PI3K or JNK expression, augmented the magnitude of apoptosis in ST cells. The suppression of MAPK or Ral pathway, and overexpression Ral expression played no role in apoptosis. The data suggest that PI3K/Akt or JNK may function downstream of aberrant Ras for the induction of apoptosis.

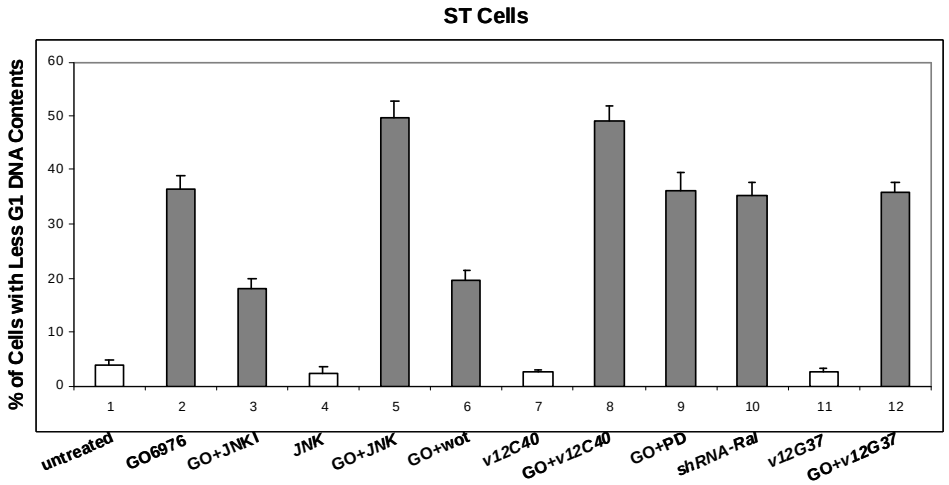

Figure 4. Induction of apoptosis in NF1 deficient ST cells infected with various Ras loop mutant genes or treated with different inhibitors. GO: GO6976; JNKI: JNK inhibitor; *JNK*: *JNK* inserted in a retroviral vector; wot: wortmannin (PI3K inhibitor); and PD: PD98059 (MAPK inhibitor).

Overall, in the funding period, a manuscript is submitted and another manuscript is being prepared to present this group of the data.

Personnel Involved in This Project:
Dr. C. Chen
Dr. J. Guo
Dr. L. Luo
Dr. L. Chen

KEY RESEARCH ACCOMPLISHMENTS

In this proposal, we hypothesize that *therapeutic intervention into crucial intracellular signaling, such as in the Ras pathway, can induce apoptosis in NF1, especially in NF1-deficient MPNST. Such an apoptotic process is regulated in the context of other regulatory influences, among them, suppression of PKC activity*. Based on our hypothesis, two specific Aims were proposed--aim 1: to determine which PKC isoform(s), once being suppressed, is/are responsible for induction of Ras-mediated apoptosis in NF1-deficient cells; and aim 2: to determine whether and how JNK functions as Ras downstream effector to transmit the apoptotic signals in NF1-deficient cells.

The data generated from this research project provided the important information indicate that:

1. Aberrant Ras in NF1 deficient cells, together with loss of PKC, are synthetically lethal.
2. After searched different location of each PKC isoform gene and selected the most effective *shRNA* sequence for each PKC isoform, we identified that the co-suppression of PKC α and β are responsible for the induction of apoptosis in *NF1* deficient cells.
3. Ras downstream effectors JNK and PI3K/Akt appear to participate in transmitting this apoptotic signaling to execute cell death program in NF1 deficient cells.
4. Ras loop mutant genes were successfully infected into *NF1* deficient or proficient cells. The results generated from these transfectants further support that JNK and PI3K/Akt play the important roles in this synthetic lethal interaction in *NF1* deficient cells.
5. We now successfully made a construct carrying *NF1* effector domain gene and tagged with Flag. After transfected with the *NF1* effector domain, *NF1* deficient cells (ST or sNF) were not susceptible to downregulation of PKC α and β-mediated apoptosis.

Overall, with the aids of these reagents and our results, it will give us further opportunity to apply for continuation funding support and hope that the knowledge generated from our current and future studies will help developing new strategies for the effective treatments.

REPORTABLE OUTCOMES

1. Chen L, Luo L. & Chen C Synthetic lethal interaction between aberrant Ras and loss PKC α/β in NF1 deficient cells. submitted.
2. Chen L, Du E & Chen C Characterization of synthetic lethality induced by hyperactive Ras and loss of PKC in NF1 deficient cells. in preparation.

CONCLUSIONS

One long-standing dogma of anti-tumor therapies has been to selectively target rapidly-dividing cells. However, this rationale is not satisfactory, because some dividing cancer cells (such as NF1 deficient cells) are relatively resistant to anti-neoplastic therapies. In recent years, the molecular mechanisms regulating apoptosis have been investigated and evidence has emerged, indicating that the perturbation of the signaling cascades mediating apoptosis, as a result of tumor development, regulates the sensitivity of malignant cells to pro-cytotoxic treatment. Therefore, we hypothesize that therapeutic intervention into crucial intracellular signaling can elicit apoptosis in NF1. Such an apoptotic process is regulated in the context of other regulatory influences, among them, suppression of PKC activity. Our data generated from this application indicate that concurrent suppression of PKC alpha and beta induces apoptosis in NF1 deficient cells, which is not harmful to NF1 proficient cells. We also identified that PKC alpha and beta play important roles in the induction of apoptosis in NF1 deficient cells. In the apoptotic process, the mitotic exit checkpoint was activated by Ras signaling in NF1 deficient cells, which may contribute to the induction of apoptotic crisis. Overall, our data generated from this project support our hypothesis that hyperactive Ras, together with loss of PKC, are synthetically lethal. However, the continuation to elucidate the molecular mechanism of how abnormal Ras/NF1 signaling cooperates with PKC suppression to induce apoptosis is undoubtedly required.

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Synthetic Lethal Interaction between Aberrant Ras and Loss of PKC α/β in *NF1* Deficient Cells

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Running Title: Synthetic Lethality Induced by PKCs and Hyperactive Ras

Abstract

Emerging evidence indicates that suppression of protein kinase C (PKC) and Ras mutations are synthetically lethal. *NF1* mutations render hyper Ras signaling, which is a main cause of the development of neurofibromatosis. Although the effort has been made, the prognosis of malignant peripheral nerve sheath tumor remains dismal, because of the lack of effective treatments. Here, using *shRNAs* or PKC inhibitor, we demonstrate that the concurrent suppression of PKC α and β induces ST cells (Schwannoma) to undergo apoptosis. In this apoptotic process, p73 and caspase 3 are activated to initiate apoptosis. However, the co-knockdown PKC α and β plays no role in PC12 cells that expressing a normal Ras. Thus, our study demonstrates that PKC α and β are necessary for sustaining the homeostasis in cells containing a hyperactive Ras. The abrogation of these two isoforms switches on the p73-regulated apoptotic machinery via the activation of caspase 3.

Key words: PKC isoforms, Ras, p73, apoptosis

Introduction

PKC, a family of serine/threonine protein kinases, consists of more than 12 isoforms that differ in their structures, cellular functions and tissue distributions (Nishizuka *et al*., 1995; Gutcher *et al*., 2003; Spitaler and Cantrell, 2004). PKC α, β_I, β_{II} and γ are categorized as the conventional or classic PKC isoforms that are calcium- and diacylglycerol (DAG)-dependent for the activation, while isozymes of unconventional PKC subgroup (PKC δ, ε, η and θ) are independent of calcium for their functions. The atypical PKC isozymes (PKC ζ and λ/ν) require neither DAG nor calcium for being activated. The structural diversity and different tissue distributions render distinct specificities of PKC isozymes. Therefore, PKC isozymes differentially regulate various cellular signaling pathways and further dictate different biological outcomes, including apoptosis. Recently, with the availability of small hairpin RNA (*shRNA*) and other genetic means to disrupt individual PKC isoform *in vitro* and *in vivo*, studies have shown that PKC isoforms are either pro- or anti-apoptotic, depending upon cell types, stimuli or contexts within signaling pathways (Matassa *et al.*, 2001; Gutcher *et al.*, 2003; Brodie and Blumberg, 2003;Reyland, 2007).

p53 exerts its tumor suppression function through controlling cell cycle checkpoints and regulating apoptosis (Levine *et al*., 1997). p73 and p63 belong to p53 family and share homology with p53 in not only their sequence but also their transactivation, DNA-binding and oligomerization functions. These two p53-related molecules have been shown to be able to activate p53-responsive genes in a p53-independent fashion. Studies have shown that p73 is involved in the cellular response to DNA damage or genotoxic stress (Jost *et al*., 1997; Yang *et al*., 2002). In response to DNA damage, the activated PKC δ binds to and phosphorylates p73, leading to apoptosis (Ren *et al*., 2002).

Ras family consists of a group of small GTPases (including three major members: Ha-, Ki and N-Ras) that, via governing various downstream effectors, regulate diverse cellular biological processes, including proliferation, differentiation, motility, transformation and apoptosis. In about 30% of human malignancies, Ras proteins are mutated. The active, GTP-bound form of Ras interacts with its downstream effectors and stimulates their activities. The balance among these intracellular signaling pathways is a key element to determine the fate of cells. In recent

years, it has been demonstrated that, under conditions of stresses (such as downregulation of endogenous PKC activity, loss of matrix adhesion, or tamoxifen treatment), hyperactive Ras in lymphocytes or fibroblasts is able to promote apoptosis (Chen *et al*., 1998a; Xia *et al*., 2007). Apoptosis initiated by mutated Ras appeared to involve multiple downstream signaling pathways (Chen *et al*., 1998b). The effort to determine the downstream effectors of Ras in transmitting pro-apoptotic signaling has been made. Overexpression of Raf renders mouse fibroblasts the susceptibility to apoptosis (Wang *et al*., 1998). Sustained activation of MAP kinase pathway in Swiss3T3 cells was linked to the induction of apoptosis (Fukasawa *et al*., 1995). Activated Ras has also been reported to be involved in stress (e.g. the UV response) related cell death, via JNK activation (Derijard *et al*., 1994). JNK has also been shown to function as apoptotic factor in apoptosis elicited by aberrant Ras (Chen *et al*., 2001).

In this study, we demonstrate that mutated Ras, together with concurrent suppression of PKC α and β, are apoptotic to the cells. In this apoptotic process, p73 and caspase 3 are activated for the induction of apoptosis in *NF1* deficient ST cells.

Results

Concurrent suppression of PKC α and β induces apoptosis in the cells with aberrant Ras signaling

Ras governs multiple downstream pathways, such as Raf/ERK/MAPK, PI3K/Akt, JNK/p38 and Ral/Rho. The *NF1* gene encodes neurofibromin (a Ras-GAP) that is highly expressed in developing neural cells and loss of function mutations in *NF1* gene results in deregulation of Ras signaling, which contributes to cancer development. The status of Ras activity in the cells was measured by the Active Ras Pull-Down and Detection kit. The baseline level of GTP bound Ras was detected in PC12 cells (rat pheochromocytoma) in which Ras signaling is intact. In comparison, a high amount of GTP bound Ras was revealed in ST cells (**Fig. 1a**). Subsequently, the activation status of Ras downstream effector pathways were examined by immunoblotting (**Fig. 1b**). ERK1/2 and Akt in ST cells, but not in PC12 cells, were phosphorylated. JNK was not activated in both cells.

GO6976 is a PKC inhibitor that is relatively specific to PKC α and β. The effect of GO6976 on the induction of apoptosis was tested by DNA fragmentation and Annexin V-FITC apoptotic detection assays (**Figs. 2a** and **b**). After treated the cells with the inhibitor for 48 h, more than 30% of ST cells underwent apoptosis, and very low numbers of PC12 or untreated ST cells were apoptotic. The data suggest that concurrent suppression of PKC α and β might be responsible for the induction of apoptosis in ST cells.

To study the molecular mechanisms of the synthetic lethal interaction between aberrant Ras and loss of PKC α plus β, the *shRNAs* against each phorbol ester-dependent PKC isoform were selected to identify their effects on the induction of apoptosis in *NF1* deficient ST cells. The *shRNAs* targeting *PKC α, β, δ, ε, η or θ* and control scrambled *shRNA*s were transiently infected into STcells. Forty-eight hours later, the protein expressions of these isoforms were examined by immunoblotting (**Fig. 3a**). The *shRNAs*, but not the scrambled *shRNAs*, blocked the expressions of targeted PKC isoforms in the cells. The level of the suppression of each PKC isoform by the *shRNA* was quantified. The expression of the isoforms of PKC in PC12 cells after infected with the *shRNAs* was also examined and similar results were obtained (data not shown). To confirm the knockdown effect by the *shRNAs* at gene level, Real-time PCR was performed (**Fig. 3b**). The

results were consistent with that shown in immunoblotting, in which the *shRNAs* effectively knocked down the gene expression of the targeted PKC isoforms. In addition, each *shRNA* did not interfere with the expression of other PKC isoform genes.

To further test the role of PKC α and β isoforms in the initiation of this apoptotic process and to confirm that the *shRNAs* did not have the off-target effect, the *shRNA*s targeting *PKC α, β* and corresponding scrambled *shRNAs* were transiently infected into PC12 and ST cells. The blockade of the expression of these two PKC isoforms was examined by immunoblotting (**Fig. 4a**). The level of PKC α or β was dramatically reduced by the corresponding *shRNA*. However, *shRNA-PKC*α and scrambled *shRNA* did not interfere with the expression of PKC β and *vice versa*. The relative expression levels of PKC α and β were measured. The induction of apoptosis after the co-suppression of PKC α plus β was tested using DNA fragmentation assay (**Fig. 4b**). The concurrent suppression of PKC α and β isozymes caused more than 30% of ST cells to undergo apoptosis, which was not apoptotic to PC12 cells. To confirm this, Annexin V-FITC apoptosis detection assay was used. After the concurrent suppression of PKC α and β by the *shRNA*s, a similar result was obtained (data not shown). It appears that genetically knockout of PKC α and β by the *shRNAs* is apoptotic to ST cells.

p73 is phosphorylated in ST cells during the apoptotic process

p73 is a member of p53 tumor suppressor family and involved in the regulation of apoptosis upon genotoxic stress or after exposure to apoptotic stimuli (Whelan et al., 1998; Agami et al., 1999; Costanzo et al., 2002; Gong et al., 1999; Gonzalez et al., 2003; Irwin et al., 2003). Studies demonstrated that p73 activation is able to initiate apoptosis in different types of cells in response to DNA damage (Ren et al., 2002; Reyland 2007). Thus, the expression of p73 in the cells was analyzed by immunoblotting (**Fig. 5a**). An increased amount of p73 was detected by the antibody in the lysate from ST cells co-infected with shRNA-PKC α and β, but not from PC12 cells co-infected with the same shRNAs, or untreated PC12 and ST cells.

Studies showed that p73 is phosphorylated at serine/theonine residues during apoptosis (Ren *et al.*, 2002; Gonzalez *et al*., 2003). Since p73 was upregulated in ST cells after co-suppressing PKC α and β, we then tested whether p73 was phosphorylated in our experimental setting. After co-infected with *shRNA-PKC*α plus *β*, the cells were subjected to immunoprecipitation with

anti-p73 antibody and subsequently, the immunoprecipitates were immunoblotted with the anti-phosphoserine antibody (**Fig. 5b**). The phosphorylated p73 was detected only in ST cells after knocking down the two PKC isoforms, but not in PC12 cells. To further evaluate the necessity of p73 for the initiation of this apoptotic process, *shRNA* directed to *p73* was introduced into ST cells after concurrently suppressing PKC α and β. To define the efficiency of triple knockdown of these molecules, the expression of p73, PKC α or β, after infected three *shRNAs* into the cells, was analyzed by immunoblotting. The *shRNAs* sufficiently inhibited the expression of these molecules (data not shown). Subsequently, the occurrence of apoptosis was assay by DNA fragmentation assay (**Fig. 5c**). The suppression of p73 blocked the apoptotic process in ST cells, indicating that p73 is a player in the induction of this cell death process.

Caspase 3 is activated for the execution of apoptosis in ST cells following the co-knockdown of PKC α and β.

Caspase family members are cysteine proteases that play important roles in apoptosis. Some caspase family members have been shown to be activated in cells expressing oncogenic *ras* for the induction of apoptosis (Chen, *et al.*, 2001). To determine if caspase 3 was activated in our experimental setting, the cleavage of caspase 3 was tested by immunoblotting. The active, cleaved form of caspase 3 was detected by the antibody in ST cells following the co-suppression of PKC α and β, which was absent in the PC cells (**Fig. 6a**). To further determine whether caspase 3 activity was required for the induction of apoptosis, DNA fragmentation assay was performed (**Fig. 6b**). Again, ST cells underwent apoptosis following co-knocking down PKC α and β. However, the addition of Z-DEVDfmk (a caspase 3 inhibitor) blocked the induction of apoptosis in the cells. However, the incomplete suppression of the apoptotic process by caspase 3 inhibitor suggests the involvement of other caspase family members or apoptotic factors.

Discussion

Study of Ras oncogene has been the popular theme in the field of cancer research in light of the pivotal roles of Ras in the regulation of diverse cellular activities, such as proliferation, differentiation, senescence and apoptosis. In the process of tumorigenesis, it is critical for hyperactive Ras to maintain the balance among deregulated downstream effectors and to properly coordinate with other intracellular signals. Disruption of one or more of these signaling pathways would perturb the balance in cells harboring a mutated Ras or Ras signaling and further trigger an apoptotic crisis. Since the *NF1* gene encodes neurofibromin (a Ras GAP) that is highly expressed in developing neural cells and loss of function mutations in *NF1* gene results in deregulation of Ras signaling, which contributes to cancer development. Whereas loss of function of neurofibromin and subsequent upregulation of Ras pathway have received considerable attention from perspectives of cancer etiology, the ability of Ras to induce apoptosis and it therapeutic perspectiveness in NF1 cells remain relatively unexplored. In our current investigation, using the *shRNA*s against PKC isoforms, we identified a regulatory network among PKC isoforms for the induction of apoptosis initiated by aberrant Ras in ST cells. We demonstrated that after the concurrent suppression of PKC α and β, p73 and caspase 3 were activated for the induction of apoptosis. This apoptotic signaling was more effectively activated when PKC α and β were concurrently suppressed. Thus, our study not only indicates that hyperactive Ras, together with loss of PKC α and β, is synthetically lethal.

Mutational activation of *ras* genes is a key event in human cancer development. During transformation process, persistent increases in Ras activity upregulate various downstream effector pathways, leading to the phosphorylation and activation of pro-growth transcriptional factors (Barbacid, 1987; Lowy *et al*., 1993; McComick *et al*., 1993). Despite its central involvement in cell growth, differentiation and tumorigenesis, activated Ras, under certain circumstances, is able to initiate apoptosis. Numerous studies have highlighted the roles of Ras in the regulation of apoptosis (McNeill *et al*., 1999). In particular, it was observed that treatment with PKC inhibitors can induce apoptosis in various types of cells overexpressing oncogenic *v-Ha* or *Ki-ras* (Chen *et al*., 1998a; Chen *et al*., 1998b; Xia *et al*., 2007). Here, we identified that the concurrent suppression of PKC α and β are critical for the induction of apoptosis in neuronal cells with aberrant Ras signaling. Since *NF1* deficient neurofibromatosis often express

hyperactive Ras activity, targeting different PKC isoforms would be an ideal strategy for developing new cancer therapeutics, because inhibition of PKC is not harmful for surrounding normal cells or tissues.

PKC isoforms are serine/threonine protein kinases that are structurally distinct and functionally diverse. There is functional redundancy among PKC isozymes for restoring normal physiological status when one or more PKC isozymes are disabled. The functions of PKC isoforms have been shown to be rather controversial, which often depend on different cellular contexts or types of stimuli. For example, studies demonstrated that PKCα, β, δ, ε, ζ could either positively or negatively regulate either tumorigenesis or apoptosis, indicating the functional complexity of PKC isozymes (Gutcher *et al*., 2003; Spitaler *et al*., 2004). Experiments have also shown the interconnection between PKC and Ras signaling pathways (Kampfer *et al*., 2001; Rusanescu *et al*., 2001). Upon mitogenic stimulation, the SH2 binding sites of PKC are phosphorylated, which in turn recruits Grb2/SOS complex and activates Ras pathway in T lymphocytes (Kawakami *et al*., 2003). PKC has also been indicated in the negative regulation of Ras signaling via modulating Ral activity (Rusanescu *et al*., 2001).

p73 is a structural and functional homologue of p53, and participates in apoptosis initiated by DNA damage stimuli. Studies demonstrated that nuclear c-Abl was involved in p73-mediated apoptosis induced by genotoxic agents (Walworth *et al*., 1993; Agami *et al*., 1999). In this process, the nuclear c-Abl interacts with p73 and stimulates p73-regulated signaling. It is also known that in response to ionizing radiation or cisplatin, p73 functions as a key element in apoptotsis (Ren *et al*., 2002). The present study shows that the co-knockdown of PKC α and β activated p73 and caspase 3 during the apoptotic process. However, the inhibition of caspase 3 only partially blocked the process of cell death. Thus, our finding does not exclude the possibility that other caspase family members or apoptotic factors, besides caspase 3, are involved in the apoptotic process in the current experimental setting. We also do not exclude the possibility that the partial suppression of apoptosis is due to the side effect of the inhibitor.

In summary, our study shows that the concurrent suppression of PKC α and β only induces ST cells to undergo apoptosis, suggesting that hyperactive Ras is an essential player in this apoptotic pathway. Ras and PKC are important intracellular signal transducers for differentiation and proliferation in many types of cells. Aberrant Ras or PKC abnormality alone is compatible

with cell viability. However, the data from our experiments suggest that aberrant Ras signaling, together with loss of PKC α plus β, severely perturb crucial survival signaling pathways, which elicits an apoptotic crisis in the cell. With increasing attention to search new strategies to treat malignant peripheral nerve sheath tumors, our study provides the evidence for developing such therapeutic strategies that preferentially kill *NF1* deficient cells at clinically achievable doses, with effective indices that are higher than those of classic cytotoxic drugs.

Materials and methods

Cells and reagents

Human NF1 deficient ST and rat PC12 cells were obtained from ATCC (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagles's medium supplemented with 10% heat-inactivated Fetal Bovine Serum (Atlanta Biologicals), 100 units/ml penicillin, 100µg/ml Streptomycin (Invitrogen). GO6976 was purchased from EMD. Antibodies against PKC α, β and Ras were purchased from BD. Antibody against p73 was from Santa Cruz Biotechnology. The anti-Phosphotyrosine or anti-Phosphoserine antibodies were obtained from Millipore and Sigma, respectively.

The oligonucleotides containing small interference (si) RNA sequences targeting different Protein Kinase C isozymes were ligated to a lentiviral small-hairpin (sh) RNA expression vector pLentiLox3.7. The sequences of the siRNAs are: 5'-gaacgtgcatgaggtgaaa-3' and 5'-tctgctgctttgttgtaca-3' for murine PKC α and PKC β, 5'-ggctgtacttcgtcatgga-3' and 5'-caggaagtcatcaggaata-3' for human PKC α and PKC β. The siRNA sequence for p73 is as reported (Chau *et al*., 2004). The plasmids expressing wild type *PKC δ* (*WT-PKCδ*) and constitutively-active *PKC δ* (*CAT-PKCδ*) were generously gifted from Dr. J. Soh (Inha University, South Korea). *v-Ha-ras* was inserted into a retroviral MSCV vector (Clontech Laboratories). FuGene 6 transfection reagent (Roche Applied Science) was used for transfections.

DNA fragmentation analysis

A flow cytometric analysis was performed using a FACScan (BD Biosciences). The data analysis was performed using the Cell-Fit software program (BD Biosciences). Cell-Fit receives data from the flow cytometer and provides real-time statistical analysis, computed at one second intervals, and also discriminates doublets or adjacent particles. Cells with sub-G_0-G_1 DNA contents after staining with propidium iodide were counted as apoptotic cells. In brief, following treatments, cells were harvested and then fixed in 70% cold ethanol. Afterwards, cells were stained with 0.1 mg/ml propidium iodide containing 1.5 mg/ml RNase. DNA contents of cells were then tested by a Becton Dickinson FACScan machine.

Annexin V-FITC apoptosis detection assay

After treatments, cells were prepared and stained with Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) according to manufacturer's instructions. Subsequently, the samples were analyzed by a flow cytometer.

Ras activation assay

Active Ras Pull-Down and Detection kit (Thermo. Scientific, IL) was used. Positive control was generated by treating lysate extracted from NIH3T3 cells with GTPγS to activate Ras.

Immunoblot analysis

Cell lysates were separated by SDS-PAGE gel and transferred to nitrocellulose. After blocking with 5% non-fat milk for 1 hour at room temperature, the nitrocellulose was probed with antibodies and then visualized by chemilluminescence (Perkin-Elmer).

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Figure legends

Figure 1. Activation of Ras signaling pathway in ST cells. **a**. The activity of Ras was measured by its ability to bind to GTP. B. The phosphorylation status of Ras downstream effectors ERK1/2, Akt and JNK in PC12 or ST cells were determined by immunoblotting. The even loading of proteins was normalinzed by re-probing the blot with corresponding antibodies.

Figure 2. Induction of apoptosis in ST cells after treated with GO6976. **a**. The cells were treated with Gö6976 (1μM) for 48 h and then collected for DNA fragmentation. **b**. Annexin V-FITC assays. The results are representative of 5 independent experiments. The error bars are SD of 5 independent experiments (n = 5, $p < 0.05$).

Figure 3. Knockdown of phorbol ester-dependent PKC isoforms by the *shRNAs* in ST cells. **a**. Whole cell lysates from ST cells infected with the *shRNAs* or scrambled *shRNAs* were prepared and immunoblotted with corresponding antibodies. The equal loading of the samples was normalized by β-actin. **b**. mRNAs were extracted from the cells infected with the *shRNAs* targeting various PKC isoforms and cDNAs were synthesized for real-time PCR analysis of the gene expressions of these PKC isoforms. The error bars represent SD (standard deviation) from three independent experiments. P values <0.05 were considered significant.

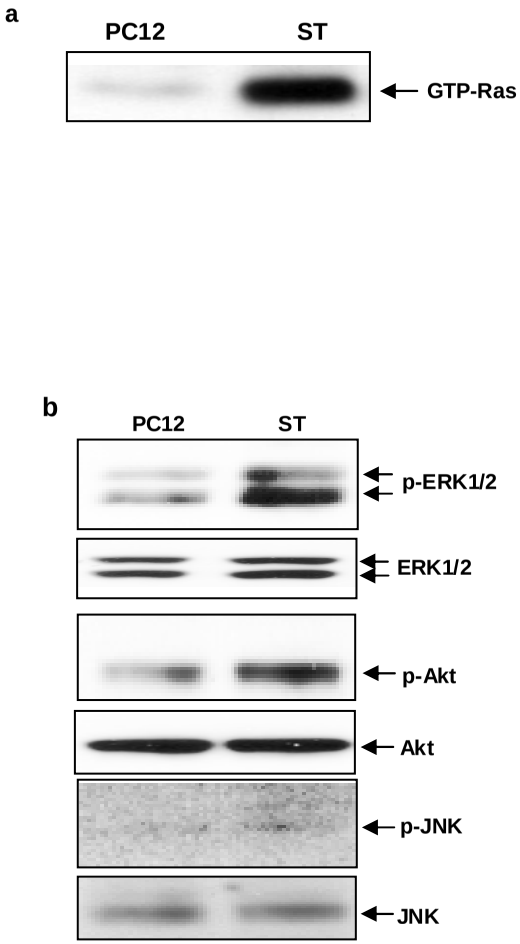
Figure 4. Induction of apoptosis in ST cells after co-knocking down PKC α and β. **a**. After transiently infected *shRNAPKCα* or *β* for 48 h, PC12 and ST cells were lysed and immunoblotted with the antibodies. The cells infected with the lentiviral vector carrying scrambled *shRNA* were used as control. The results are representative of three independent experiments. β-actin was used as loading control. **b**. Following co-inhibited PKC α and β with the *shRNA*s, the cells were subjected to Annexin V-FITC analysis. The error bars represent SD (standard deviation) over five independent experiments.

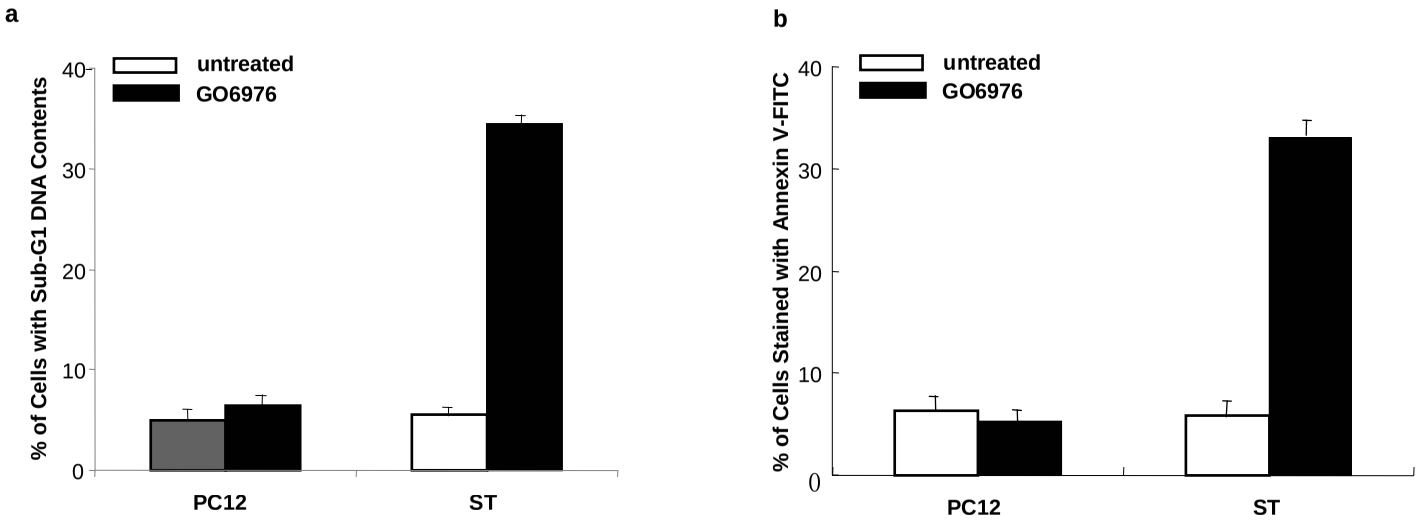
Figure 5. p73 is upregulated upon co-knockdown of PKC α and β in ST cells. **a**. Following the co-infection with *shRNAPKCα* and *β*, cell lysates were prepared and immunoblotted with anti-p73 antibody. *β*-actin was used as loading control. **b**. With or without co-infection with *shRNAPKCα* and *β*, cell lysates were immunoprecipitated with anti-p73 antibody. The immunoprecipitates were then subjected to immunoblotting using the anti-phosphoserine

antibody. The input was judged by probing the same amount of immunoprecipitates with anti-p73 antibody. **c**. After infected *shRNAPKCα*, *β* and *shRNAp73*, ST cells were subjected to DNA fragmentation assay. The plot is representative of 5 independent experiments. Error bars represent SD from 5 independent experiments (n = 5, $p < 0.05$).

Figure 6. Caspase 3 is activated and required for the induction of apoptosis in ST cells. **a**. Following co-knocking down PKC α plus β, immunoblotting was conducted to detect the presence of active capase 3 using anti-caspase 3 antibody. **b**. After infected *shRNAPKCα*, *β* in the presence or absence of Z-DEVDfmk, ST cells were subjected to DNA fragmentation assay. The plot is representative of 5 independent experiments. Error bars represent SD from 5 independent experiments (n = 5, $p < 0.05$).

Figure 1a and b, Chen et al.

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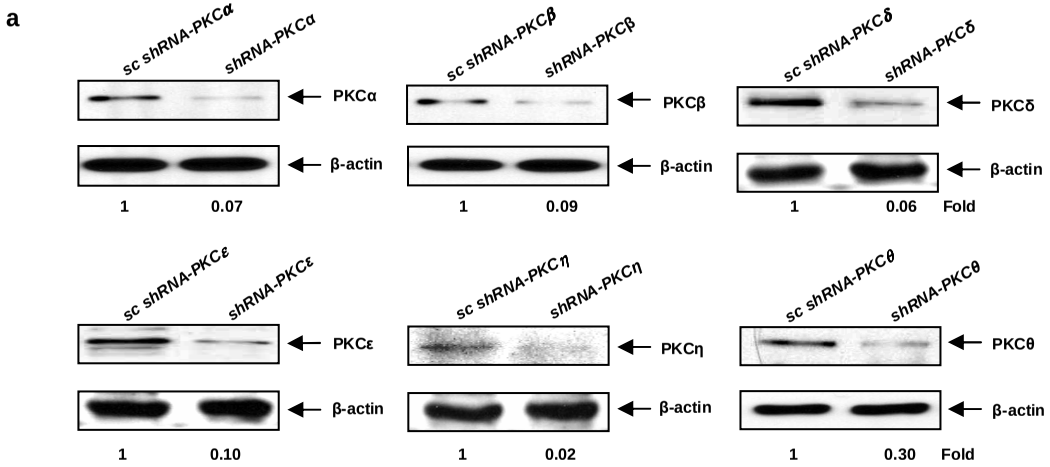
untreated
GO6976
% of Cells with Sub-G1 DNA Contents
40
30
20
10
0
PC12
ST
b
untreated
GO6976
% of Cells Stained with Annexin V-FITC
40
30
20
10
0
PC12
ST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shRNA-PKCα
PKCα
β-actin
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0.07
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shRNA-PKCβ
PKCβ
β-actin
1
0.09
sc shRNA-PKCδ
shRNA-PKCδ
PKCδ
β-actin
1
0.06
Fold
sc shRNA-PKCε
shRNA-PKCε
PKCε
β-actin
1
0.10
sc shRNA-PKCη
shRNA-PKCη
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1
0.02
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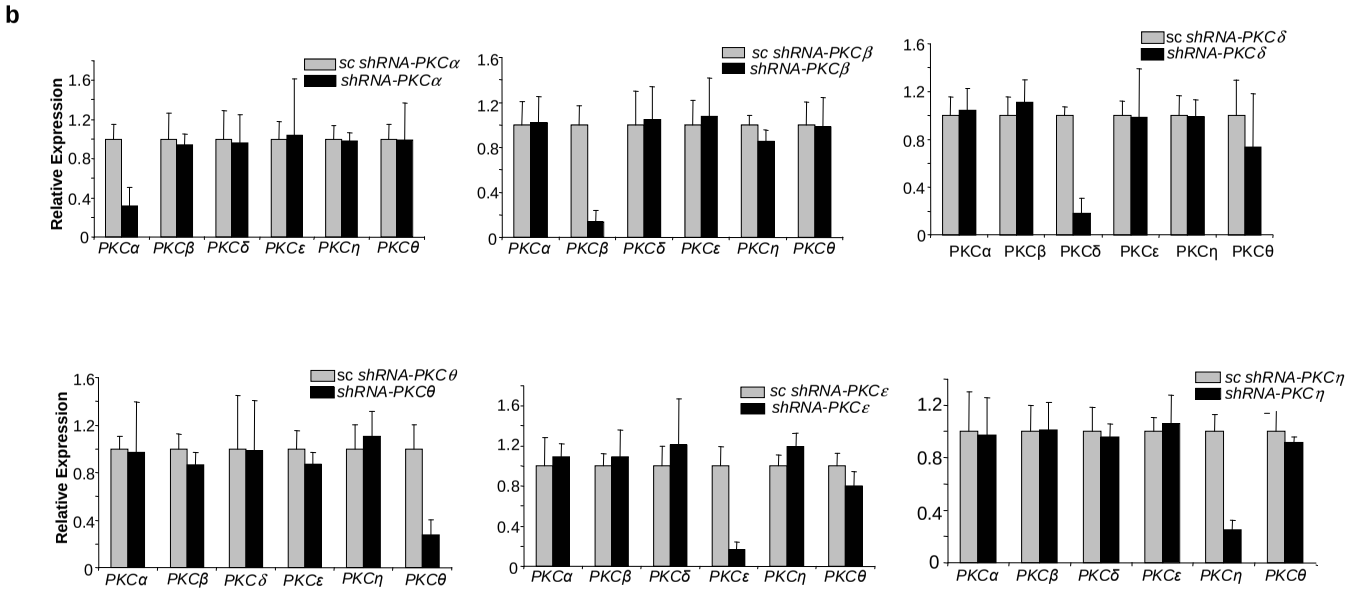
PKCθ
β-actin
1
0.30
Fold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shRNA-PKCα
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shRNA-PKCβ
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sc shRNA-PKCδ
shRNA-PKCδ
PKCα PKCβ PKCδ PKCε PKCη PKCθ
Relative Expression
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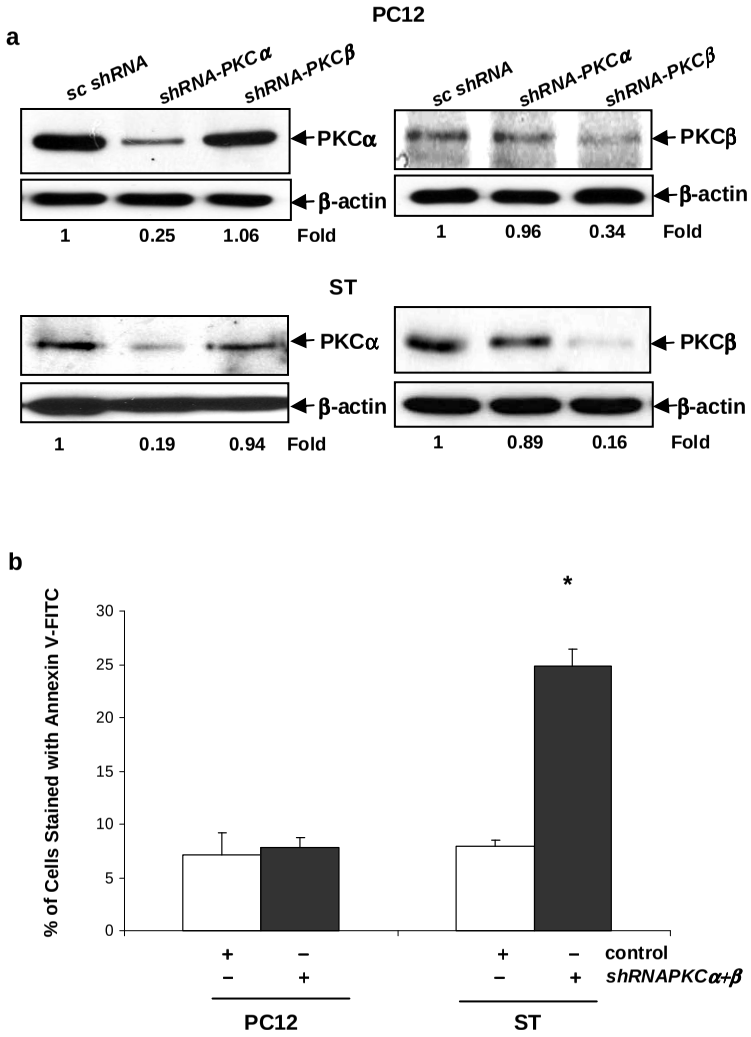
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shRNA-PKCα
shRNA-PKCβ
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0.25
1.06
Fold
PKCβ
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0.96
0.34
ST
0.19
0.94
0.89
0.16
b
% of Cells Stained with Annexin V-FITC
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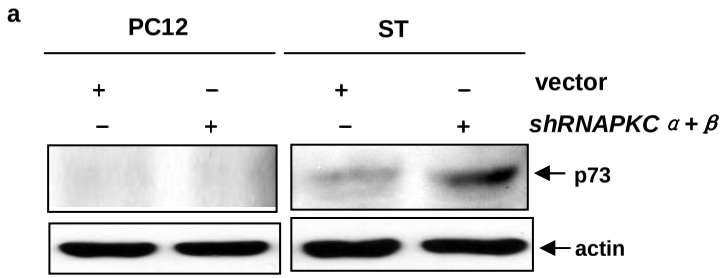
25
20
15
10
5
0
*
control
shRNAPKCα+β
PC12
ST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+ – + – vector
– + – + shRNAPKC α + β
p73
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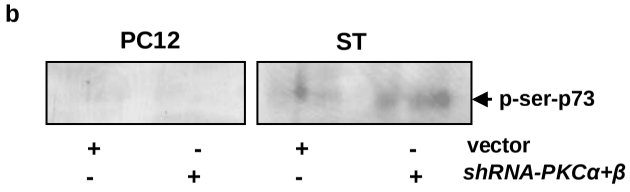
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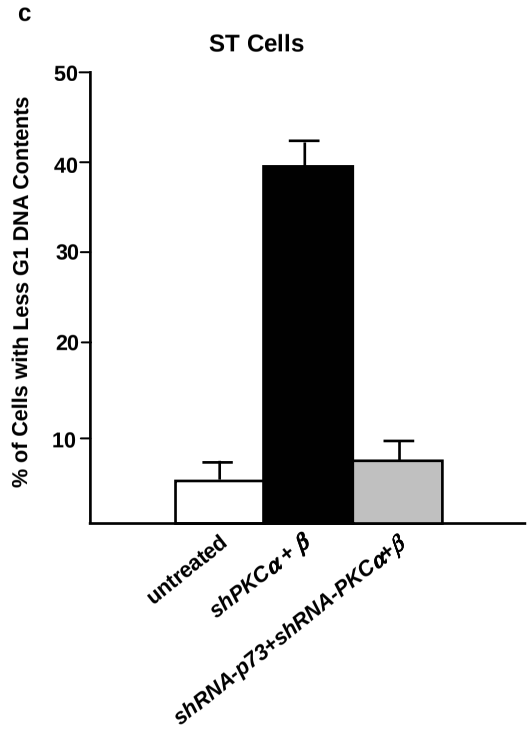
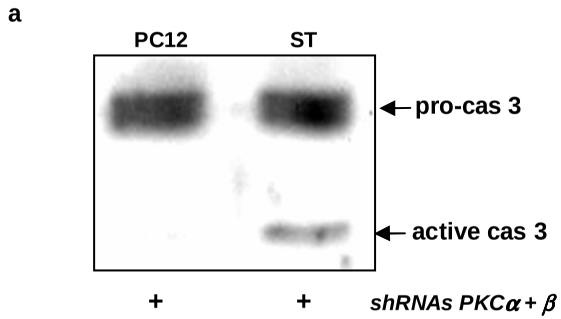
ST
p-ser-p73
+ - + - vector
- + - + shRNA-PKCα+β

Figure 5c, Chen et al.

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ST
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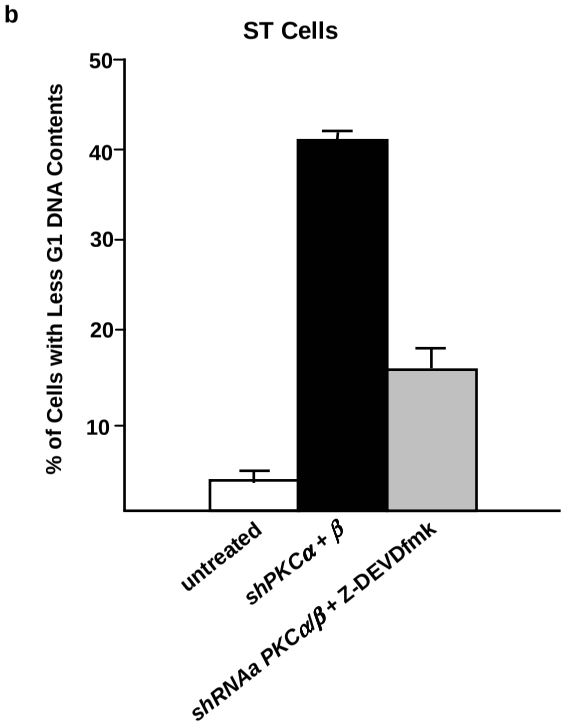
active cas 3
+
+
shRNAs PKCα + β


b
ST Cells
% of Cells with Less G1 DNA Contents
50
40
30
20
10
untreated
shPKCα + β
shRNAa PKCα/β + Z-DEVDfmk